

# Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: Role of sphingosine-1-phosphate

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**Aims** High-density lipoprotein (HDL) has been reported to have cardioprotective properties independent from its cholesterol transport activity. The influence of native HDL and reconstituted HDL (rHDL) on Stat3, the transcription factor playing an important role in myocardium adaptation to stress, was analysed in neonatal rat ventricular cardiomyocytes. We have investigated modulating the composition of rHDL as a means of expanding its function and potential cardioprotective effects.

**Methods and results** Stat3 phosphorylation and activation were determined by western blotting and electrophoretic mobility shift assay (EMSA). In ventricular cardiomyocytes, HDL and the HDL constituent sphingosine-1-phosphate (S1P) induce a concentration- and time-dependent increase in Stat3 activation. They also enhance extracellular signal-regulated kinases (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) phosphorylation. U0126, a specific inhibitor of MEK1/2, the upstream activator of ERK1/2, abolishes HDL- and S1P-induced Stat3 activation, whereas the p38 MAPK blocker SB203580 has no significant effect. Inhibition of the tyrosine kinase family Src (Src) caused a significant reduction of Stat3 activation, whereas inhibition of phosphatidylinositol 3-kinase (PI3K) had no effect. S1P and rHDL containing S1P have a similar strong stimulatory action on Stat3, ERK1/2, and p38 MAPK comparable to native HDL. S1P-free rHDL has a much weaker effect. Experiments with agonists and antagonists of the S1P receptor subtypes indicate that HDL and S1P activate Stat3 mainly through the S1P2 receptor. **Conclusion** In ventricular cardiomyocytes, addition of S1P to rHDL enhances its therapeutic potential by improving its capacity to activate Stat3. Activation of Stat3 occurs mainly via the S1P constituent and the lipid receptor S1P2 requiring stimulation of ERK1/2 and Src but not p38 MAPK or PI3K. The study underlines the therapeutic potential of tailoring rHDL to confront particular clinical situations.

## 1. Introduction

High-density lipoprotein (HDL) is known to remove cholesterol from peripheral tissues in a process known as reverse cholesterol transport. Beyond this function, HDL has been shown to exert cardiovascular protective effects in endothelial and vascular smooth muscle cells by generating a cascade of intracellular signals including the activation of PI3K/Akt, ERK1/2, p38 mitogen-activated protein kinase (MAPK), and RhoA.<sup>1–3</sup>

Several studies have demonstrated that reconstituted HDL (rHDL) represents an important therapeutic potential against cardiovascular disease.<sup>4–6</sup> rHDL typically consists of the apolipoprotein (apo) AI and phospholipids. A recent innovation has been to complement rHDL with other bioactive molecules that can extend its physiological impact.

Matsuo *et al.*<sup>6</sup> developed a novel rHDL containing apoAI, phosphatidylcholine, and sphingosine-1-phosphate (S1P) which has been shown to play a major role in HDL-induced cellular signalling.<sup>2</sup> This rHDL exerted S1P-mediated stimulatory effects on cell proliferation and tube formation in endothelial cells.<sup>6</sup>

There is little information concerning a direct protective effect of HDL or rHDL on the heart. Calabresi *et al.*<sup>7</sup> showed that HDL protects isolated rat hearts from ischaemia/reperfusion (I/R) injury by reducing the ischaemia-induced cardiac tumour necrosis factor- $\alpha$  expression. Recently, Theilmeier *et al.*<sup>8</sup> demonstrated that native, human HDL, and its sphingolipid component, S1P, protect the heart against I/R injury *in vivo*.

Surprisingly, there is no information concerning HDL-induced activation of the signal transducer and activator of transcription 3 (Stat3), despite growing evidence that Stat3 plays an important role in cardiac remodelling. Multiple

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studies have shown that the activation of Stat3 promotes cardiomyocyte survival and hypertrophy, as well as cardiac angiogenesis, indicating that Stat3 is beneficial for the heart.<sup>9,10</sup> During its activation Stat3 acquires an obligatory tyrosine phosphorylation<sup>11</sup> as well as serine phosphorylation which appears to be in some cases necessary for maximal transcriptional activity of Stat3,<sup>12</sup> while inhibitory in others.<sup>13</sup>

If rHDL is to be tailored to particular clinical effects, it is important to attribute these effects to the added molecules and define the regulatory pathways involved. In the present work, we investigated the activation of Stat3 by HDL isolated from human plasma and by rHDL containing S1P and/or apoA1 in neonatal rat ventricular cardiomyocytes. Moreover, we studied the involvement of ERK1/2, p38 MAPK, tyrosine kinase family Src (Src), PI3K, Rho-associated kinase (ROCK), phospholipase C (PLC), and protein kinase C (PKC) in HDL-induced Stat3 activation.

## 2. Methods

A detailed description of Methods is available at *Cardiovascular Research* online.

### 2.1 Cell culture

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No. 85-23, revised 1966). It was also approved by the Office Vétérinaire Fédéral and Cantonal, Geneva, Switzerland. Neonatal cardiomyocytes were isolated from 1-to-2-day-old Wistar rats ventricles by digestion with trypsin-EDTA and type 2 collagenase as we have previously described.<sup>14</sup> Cardiomyocytes were used on the third day of culture, after 16–20 h in DMEM medium depleted in FCS.

### 2.2 Western blotting

After stimulation, cells were washed with PBS and lysed. Total cell proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Afterwards, membranes were probed with specific antibodies against phosphorylated Stat3, ERK1/2, p38 MAPK, and Src and reprobed with antibodies against total Stat3, p38 MAPK, or Src. Specific bands were visualized with a chemiluminescence kit.

### 2.3 Electrophoretic mobility shift assay

Nuclear extracts were prepared and electrophoretic mobility shift assay performed as previously described.<sup>14</sup> To specify Stat3-SIE (sis-inducible element) DNA binding, polyclonal anti-Stat3 antibody or anti-Stat1 antibody were added to the reaction mixture containing the labelled probe. To confirm the specificity of the labelled SIE DNA probe, competition assay was performed using unlabelled SIE DNA sequence.

### 2.4 Preparation of HDL and of reconstituted HDL

HDL ( $d = 1.063\text{--}1.21$  g/mL) was isolated by cumulative flotation ultracentrifugation according to James *et al.*<sup>15</sup> from a plasma pool provided by healthy volunteers. Three different forms of rHDL were prepared by the cholate dialysis procedure.<sup>16</sup> rHDL A contained phospholipids and S1P; rHDL B contained phospholipids, S1P and apoA1; rHDL C contained phospholipids and apoA1. For preparations B and C, the phospholipid:apoA1 molar ratio was 100:1.

### 2.5 Statistical analysis

All values are expressed as mean  $\pm$  SEM. Differences between groups were determined using either two-tailed unpaired Student's

*t*-tests or one-way ANOVA, followed by Bonferroni's *post-hoc* test where applicable.  $P < 0.05$  was accepted as statistically significant.

## 3. Results

### 3.1 HDL and sphingosine-1-phosphate induce a time- and concentration-dependent serine and tyrosine phosphorylation of Stat3

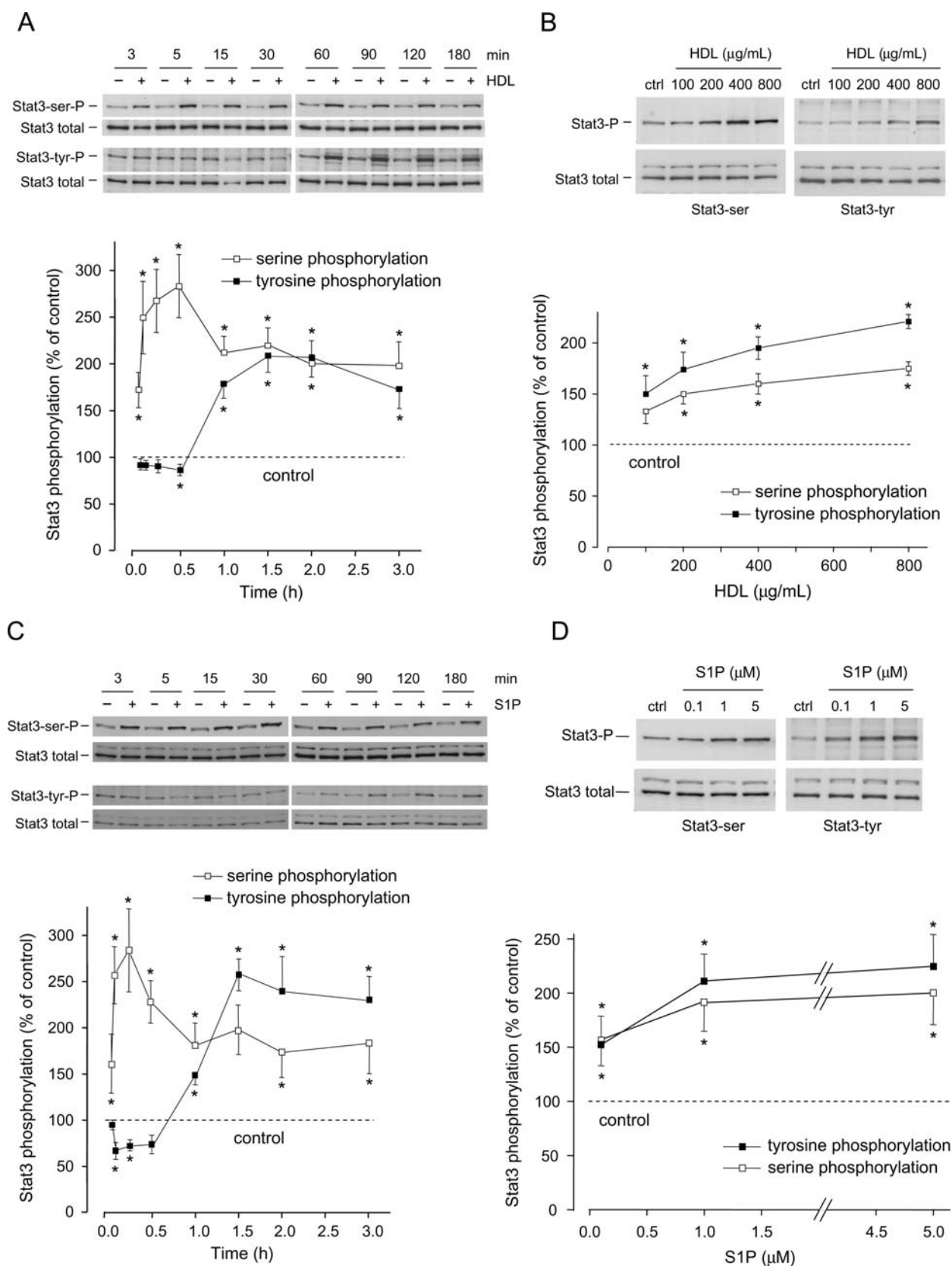
The activation of Stat3 by HDL and its constituent S1P was first investigated by measuring Stat3 phosphorylation on tyrosine and serine residues in ventricular cardiomyocytes by western blotting. As shown in *Figure 1A*, HDL (400  $\mu$ g protein/mL), corresponding to physiological extravascular concentrations,<sup>17</sup> induces Stat3 phosphorylation on tyrosine 705 as well as on serine 727 in a time-dependent manner. It promoted a significant serine phosphorylation within 3 min, reaching maximal values after 30 min. In contrast, tyrosine phosphorylation occurred only 1 h after exposition of cardiomyocytes to HDL. Both serine and tyrosine phosphorylation levels were sustained during 1–3 h of stimulation with HDL. The kinetics of S1P-induced Stat3 serine and tyrosine phosphorylation were very similar to those of HDL-induced responses (*Figure 1C*). The HDL- and S1P-induced increase in Stat3 phosphorylation was also found to be concentration-dependent, with significant responses observed after 90 min of stimulation with 100  $\mu$ g/mL of HDL (*Figure 1B*) and with 100 nM of S1P (*Figure 1D*).

### 3.2 Effect of HDL, sphingosine-1-phosphate, and different reconstituted HDL on Stat3 phosphorylation and on Stat3 DNA-binding

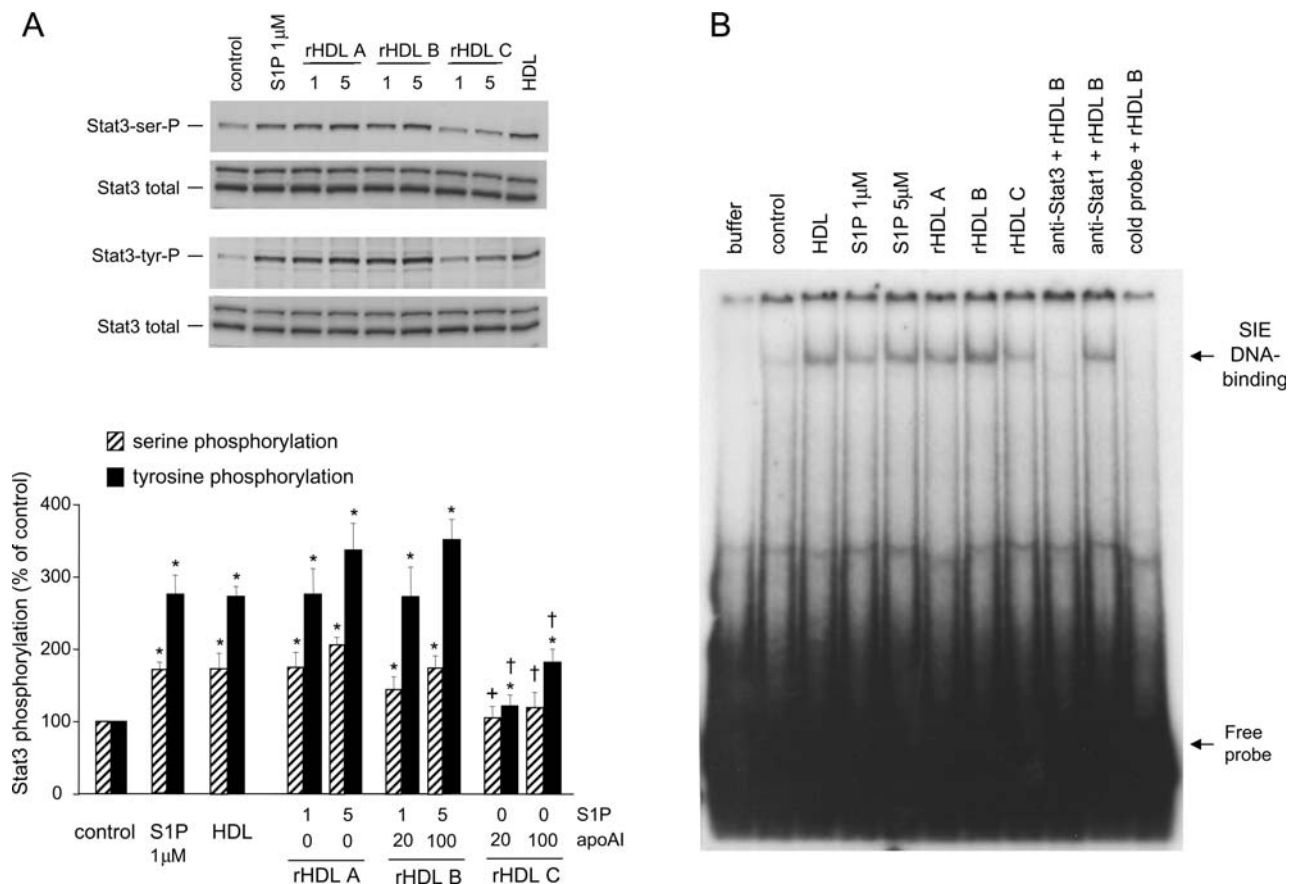
To investigate the specific role of the HDL constituents, S1P and apoA1 in HDL-induced Stat3 phosphorylation, we stimulated cells with three different rHDLs, containing, respectively, S1P alone (rHDL A), S1P+apoA1 (rHDL B), and apoA1 (rHDL C). We used two concentrations: 1  $\mu$ M S1P and/or 20  $\mu$ g/mL apoA1, and 5  $\mu$ M S1P and/or 100  $\mu$ g/mL apoA1. Cells were also stimulated with native, human HDL (400  $\mu$ g/mL) and S1P (1  $\mu$ M). As illustrated in *Figure 2A*, the two rHDLs (rHDL A and rHDL B) containing S1P (1  $\mu$ M) increased serine and tyrosine phosphorylation of Stat3 in the same way as HDL and S1P. rHDL containing higher concentrations of S1P (5  $\mu$ M) did not significantly augment the responses obtained with the lower concentration. In contrast, rHDL C which contained only apoA1 had a significantly weaker effect on Stat3 phosphorylation. Consistently, rHDL B containing S1P and apoA1 induced the same response as HDL A containing only S1P.

*Figure 2B* illustrates the Stat3 DNA-binding induced by rHDL, HDL, and S1P. Cells were incubated for 90 min with HDL (400  $\mu$ g/mL), S1P (1 and 5  $\mu$ M), and the three rHDL preparations (1  $\mu$ M S1P, 1  $\mu$ M S1P+20  $\mu$ g/mL apoA1, and 20  $\mu$ g/mL apoA1). As shown, 90 min of stimulation with HDL or S1P strongly enhanced the SIE DNA-binding of Stat3. In contrast, 5 min of stimulation with HDL or S1P had no effect on the Stat3 DNA-binding (data not shown). As for Stat3 phosphorylation, S1P and the two rHDLs containing S1P (rHDL A and B) markedly increased Stat3 DNA-binding, whereas rHDL C containing only apoA1 had a considerably weaker effect.

As shown in *Figure 2B*, the specificity of the Stat3/SIE DNA complex is demonstrated by the disappearance of this complex in the presence of anti-Stat3 antibody. In contrast, the anti-Stat1 antibody had no effect, confirming specific Stat3 binding upon HDL stimulation. During competition assay



**Figure 1** HDL and S1P induce an increase in Stat3 phosphorylation on tyrosine 705 and serine 727. (A and C) Time-dependent increase in Stat3 phosphorylation on tyrosine 705 and serine 727 induced by HDL (A, 400  $\mu\text{g/mL}$ ) and S1P (C, 1  $\mu\text{M}$ ), compared with unstimulated cardiomyocytes (control). (B and D) Cells were stimulated for 90 min with increasing concentrations of HDL (B, 100–800  $\mu\text{g/mL}$ ) or S1P (D, 0.1–5  $\mu\text{M}$ ). (A–D) Stat3 phosphorylation on tyrosine 705 (Stat3-tyr-P) and serine 727 (Stat3-ser-P) was detected by western blotting; protein loading was assessed by total Stat3. Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the control. \* $P < 0.05$  compared with control values ( $n = 3-4$ ).



**Figure 2** Activation of Stat3 by HDL, rHDL, and S1P. Cardiomyocytes were untreated (control) or stimulated for 90 min with HDL (400  $\mu$ g/mL), S1P (1 or 5  $\mu$ M) or three different rHDL containing S1P (rHDL A), S1P+apoA1 (rHDL B), or apoA1 (rHDL C). (A) Cells were incubated with two concentrations of rHDL: 1  $\mu$ M S1P and/or 20  $\mu$ g/mL apoA1 (1), and 5  $\mu$ M S1P and/or 100  $\mu$ g/mL apoA1 (5). Stat3 phosphorylation on tyrosine 705 (Stat3-tyr-P) and serine 727 (Stat3-ser-P) was detected by western blotting; protein loading was assessed by total Stat3. Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the control. \* $P$  < 0.05 compared with control values, † $P$  < 0.05 vs. rHDL A and rHDL B, + $P$  < 0.05 vs. rHDL A ( $n$  = 3). (B) Cardiomyocytes were stimulated with the three rHDL containing 1  $\mu$ M S1P and/or 20  $\mu$ g/mL apoA1. DNA-binding of Stat3 was analysed in nuclear extracts by electrophoretic mobility shift assay using labelled SIE DNA. To determine Stat3 specific DNA-binding, antibodies against Stat3 and Stat1 were used. The specificity of the labelled DNA probe was confirmed by competition assay using a 100-fold excess of unlabeled SIE DNA (cold probe). The figure shows a representative example from three experiments.

with a 100-fold excess of unlabelled SIE DNA (cold probe), the band corresponding to the binding of Stat3 to the SIE sequence disappeared, confirming the specificity of SIE DNA binding.

Our results indicate that HDL activates Stat3 mainly via its sphingolipid constituent S1P.

### 3.3 HDL and sphingosine-1-phosphate induce Stat3 phosphorylation via the S1P2 receptor

To determine which S1P receptors are involved in the Stat3 phosphorylation induced by HDL and S1P, we used several agonists and antagonists of the S1P receptors S1P1–S1P5.<sup>18–20</sup> We found that the presence of the highly selective S1P2 antagonist, JTE013,<sup>18</sup> abolished the ability of S1P to induce Stat3 serine and tyrosine phosphorylation, and significantly inhibited that of HDL (Figure 3A). In contrast, incubation of cardiomyocytes with SEW2871, an S1P1 receptor-specific agonist, and FTY720, a high-affinity agonist of S1P1, S1P3, S1P4, and S1P5 receptors, did not induce Stat3 phosphorylation, neither on serine nor on tyrosine (Figure 3B). Neither did VPC23019, a competitive antagonist of the S1P1/S1P3 receptors,<sup>20</sup> significantly affect HDL- and S1P-induced Stat3 phosphorylation (Figure 3C). Interestingly, pre-treatment of cells with pertussis toxin (PTX, 1  $\mu$ g/mL) for 18 h had no

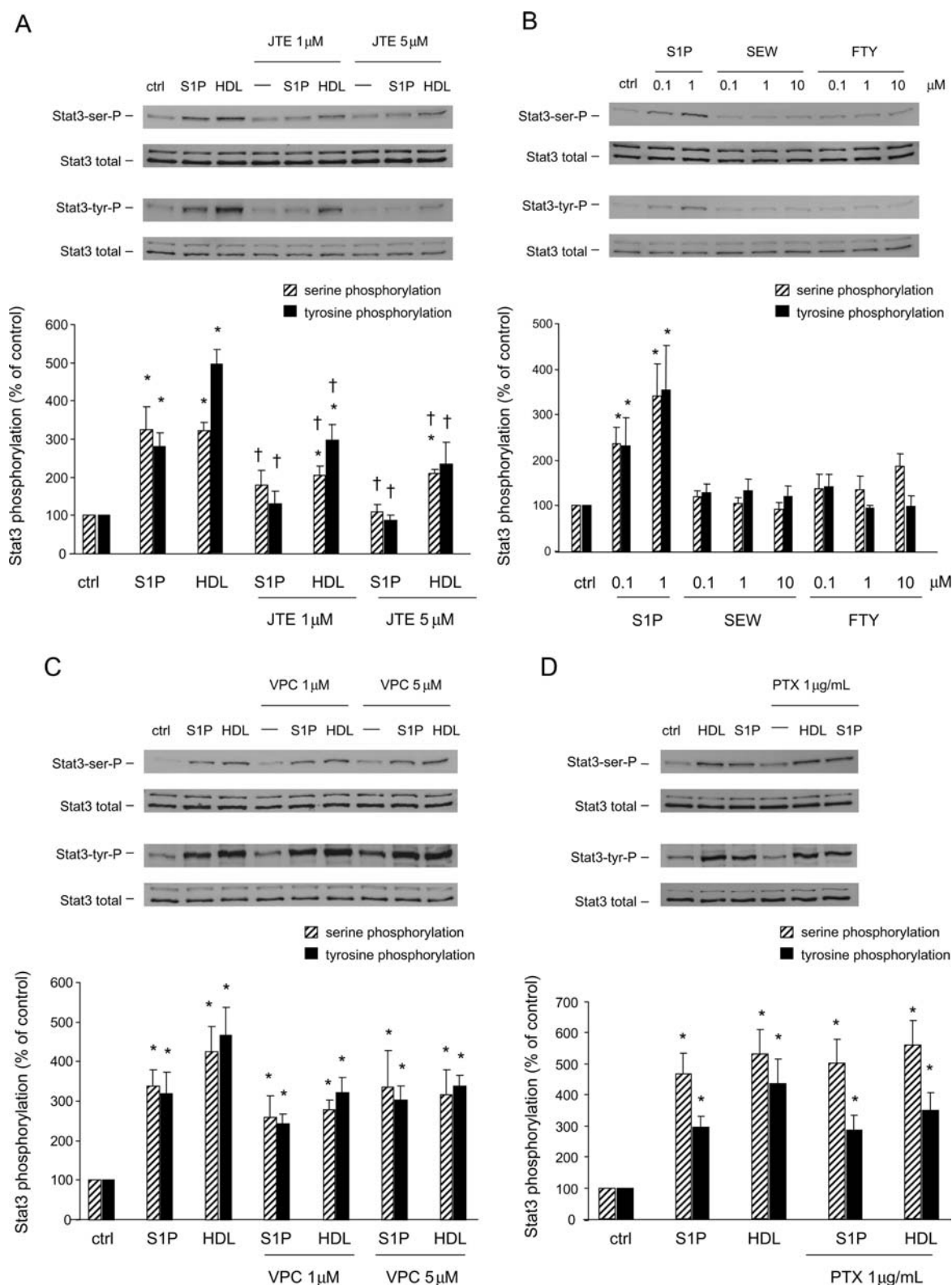
significant effect on HDL- and S1P-induced Stat3 serine and tyrosine phosphorylation (Figure 3D).

Thus our results indicate that HDL and S1P induce PTX-insensitive Stat3 phosphorylation essentially through the receptor S1P2, although in the case of HDL, a possible involvement of another receptor, with a lesser impact, cannot be excluded.

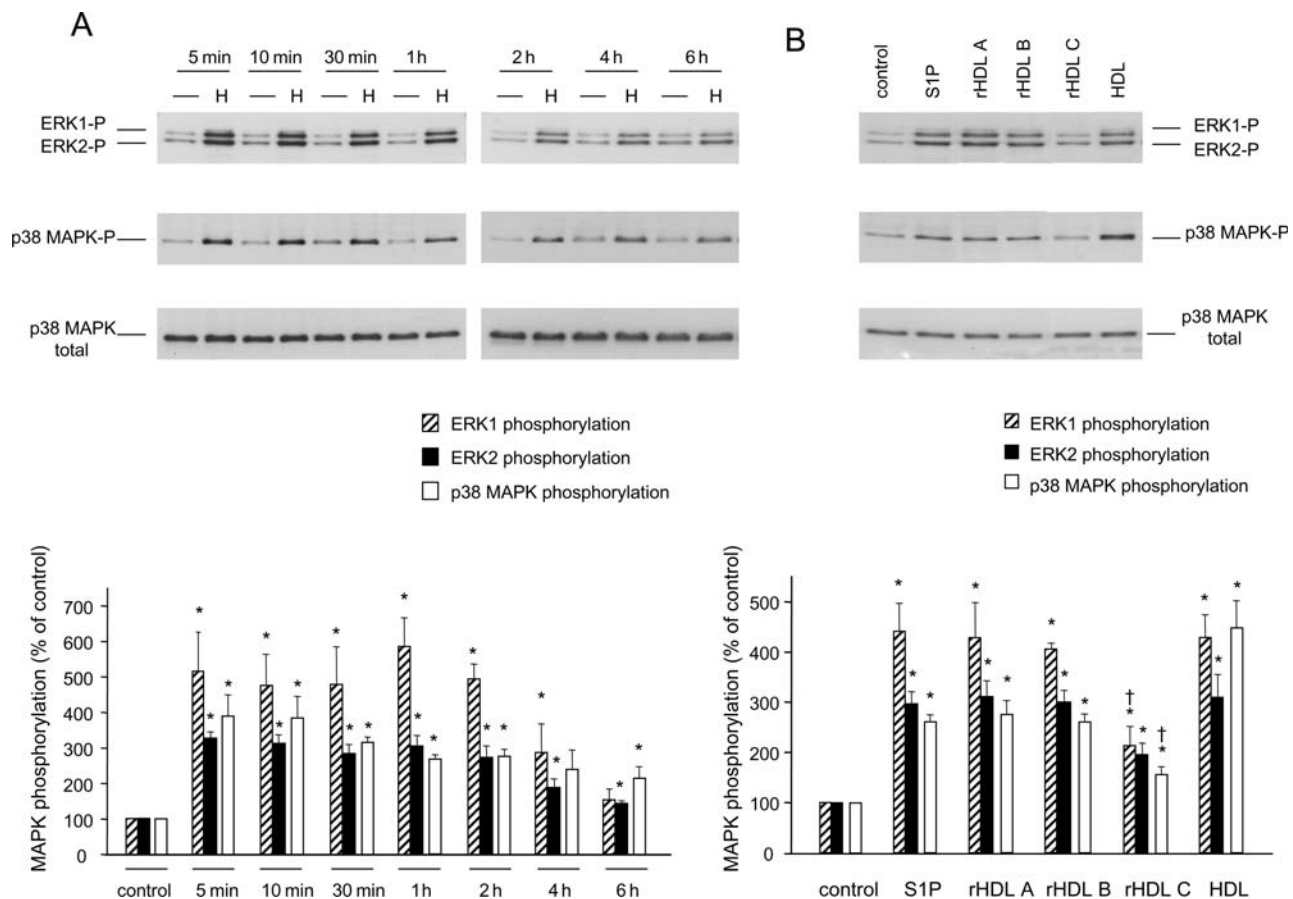
### 3.4 ERK1/2, but neither p38 MAPK nor PI3K, plays a role in HDL- and sphingosine-1-phosphate-induced Stat3 activation

Since ERK1/2 and p38 MAPK can act as modulators of Stat3 activation, we studied their role in the activation of Stat3 induced by HDL or S1P in ventricular cardiomyocytes. We first investigated the effect of HDL and S1P on the phosphorylation of ERK1/2 and p38 MAPK. As shown in Figure 4A, HDL induced a rapid and important phosphorylation of ERK1/2 and p38 MAPK with significant responses observed after 5 min. The ERK1/2 phosphorylation level remained constant during 2 h of exposition to HDL, whereas that of p38 MAPK decreased slowly after 10 min of stimulation. However, both ERK1/2 and p38 MAPK phosphorylation levels were still increased after 6 h of





**Figure 3** HDL and S1P induce Stat3 phosphorylation through S1P2. Cardiomyocytes were untreated (ctrl) or incubated with S1P antagonists or pertussis toxin, prior to stimulation for 90 min with S1P (1  $\mu$ M), S1P agonists (0.1–10  $\mu$ M), or HDL (400  $\mu$ g/mL). Stat3 phosphorylation (Stat3-P) was analysed by western blotting as illustrated by representative western blots. Equal gel loading were assessed by total Stat3. Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. \* $P$  < 0.05 compared with control values, † $P$  < 0.05 vs. S1P or HDL ( $n$  = 4–6). (A) Cells were incubated for 20 min with the specific S1P2 antagonist, JTE013 (JTE, 1 and 5  $\mu$ M) prior to stimulation with S1P or HDL ( $n$  = 6). (B) Cardiomyocytes were stimulated for 90 min with 0.1 and 1  $\mu$ M of S1P, the S1P1-specific receptor agonist SEW2871 (SEW) or the high-affinity agonist of S1P1, S1P3, S1P4, and S1P5 receptors, FTY720 (FTY) ( $n$  = 4). (C) Cells were treated for 20 min with the selective S1P1/S1P3 antagonist, VPC23019 (VPC, 1 and 5  $\mu$ M) prior to stimulation with S1P or HDL ( $n$  = 6). (D) Cardiomyocytes were incubated for 18 h with pertussis toxin (PTX, 1  $\mu$ g/mL) prior to stimulation with S1P or HDL ( $n$  = 4).



**Figure 4** HDL, rHDL, and S1P induce ERK1/2 and p38 MAPK phosphorylation. (A) Cardiomyocytes were untreated (control) or stimulated with HDL (H, 400  $\mu$ g/mL) during different time periods (5 min–6 h). (B) Cells were stimulated with the three rHDL containing 1  $\mu$ M S1P and/or 20  $\mu$ g/mL apoA1. Cells were also exposed to S1P (1  $\mu$ M) and native HDL (400  $\mu$ g/mL). (A and B) Phosphorylation of ERK1/2 (ERK1/2-P) and p38 MAPK (p38 MAPK-P) was analysed by western blotting, and protein loading was determined by total p38 MAPK. Specific bands corresponding to the phosphorylated forms of ERK1/2 and p38 MAPK were quantified by densitometry and expressed as percentage of the corresponding control. \* $P$  < 0.05 compared with control values, † $P$  < 0.05 vs. rHDL A and rHDL B ( $n$  = 3–4).

stimulation with HDL. The kinetics of S1P-induced ERK1/2 and p38 MAPK phosphorylation was very similar to those of HDL-induced responses (data not shown).

As for Stat3 phosphorylation, the rHDLs containing S1P (rHDL A and rHDL B) induced a degree of phosphorylation of ERK1/2 and p38 MAPK similar to S1P alone, whereas rHDL C containing no S1P elicited a much weaker response (see Figure 4B).

Experiments with agonists and antagonists of S1P receptor subtypes indicate that, as for Stat3 activation, HDL and S1P activate ERK1/2 and p38 MAPK mainly through the S1P2 receptor (Figure 5). Indeed, the S1P2 receptor antagonist JTE013 significantly reduced Stat3 ERK1/2 and p38 MAPK phosphorylation induced by S1P or HDL (Figure 5A). In contrast, SEW2871 (S1P1 receptor agonist) and FTY720 (S1P1, S1P3, S1P4, S1P5 receptor agonist) had only a weak, non-significant effect on HDL- and S1P-induced ERK1/2 and p38 MAPK phosphorylation (Figure 5B). Similarly, the S1P1/S1P3 receptor antagonist VPC23019 did not significantly affect HDL- and S1P-induced phosphorylation of these kinases (Figure 5C).

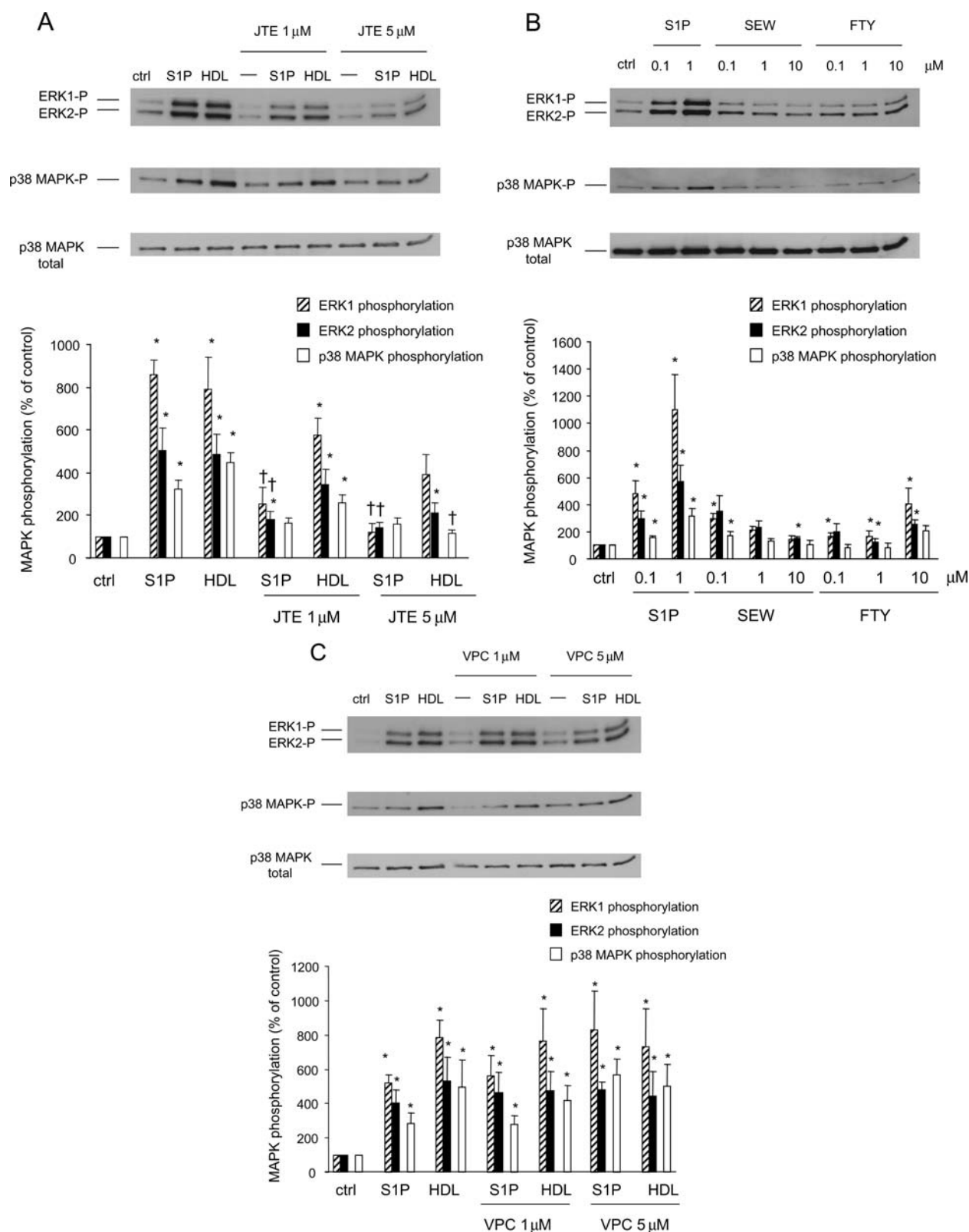
To investigate the involvement of p38 MAPK and the ERK1/2 cascade in HDL- and S1P-induced Stat3 activation, we determined Stat3 phosphorylation and Stat3 DNA-binding after 90 min of stimulation with HDL or S1P in the presence and absence of their respective inhibitors,<sup>21</sup> SB203580 and U0126. As shown in Figure 6A, blocking the ERK1/2 signalling pathway with U0126 (10  $\mu$ M), strongly reduced the HDL- and

S1P-induced serine and tyrosine phosphorylation of Stat3, whereas inhibiting p38 MAPK with SB203580 (10  $\mu$ M), had no significant effect. As for Stat3 phosphorylation, HDL and S1P-induced Stat3 DNA-binding was essentially suppressed by U0126 while it was only slightly affected by SB203580 (data not shown). Similarly, inhibition of PI3K by LY294002 had no effect on HDL- and S1P-induced Stat3 phosphorylation, neither on tyrosine nor on serine (Figure 6B). Both these inhibitors are active in our cellular model, since we observed that LY294002 abolished leukemia inhibitory factor-induced phosphorylation of Akt in ventricular cardiomyocytes, while SB203580 inhibited HDL- and S1P-induced cyclooxygenase-2 expression (data not shown).

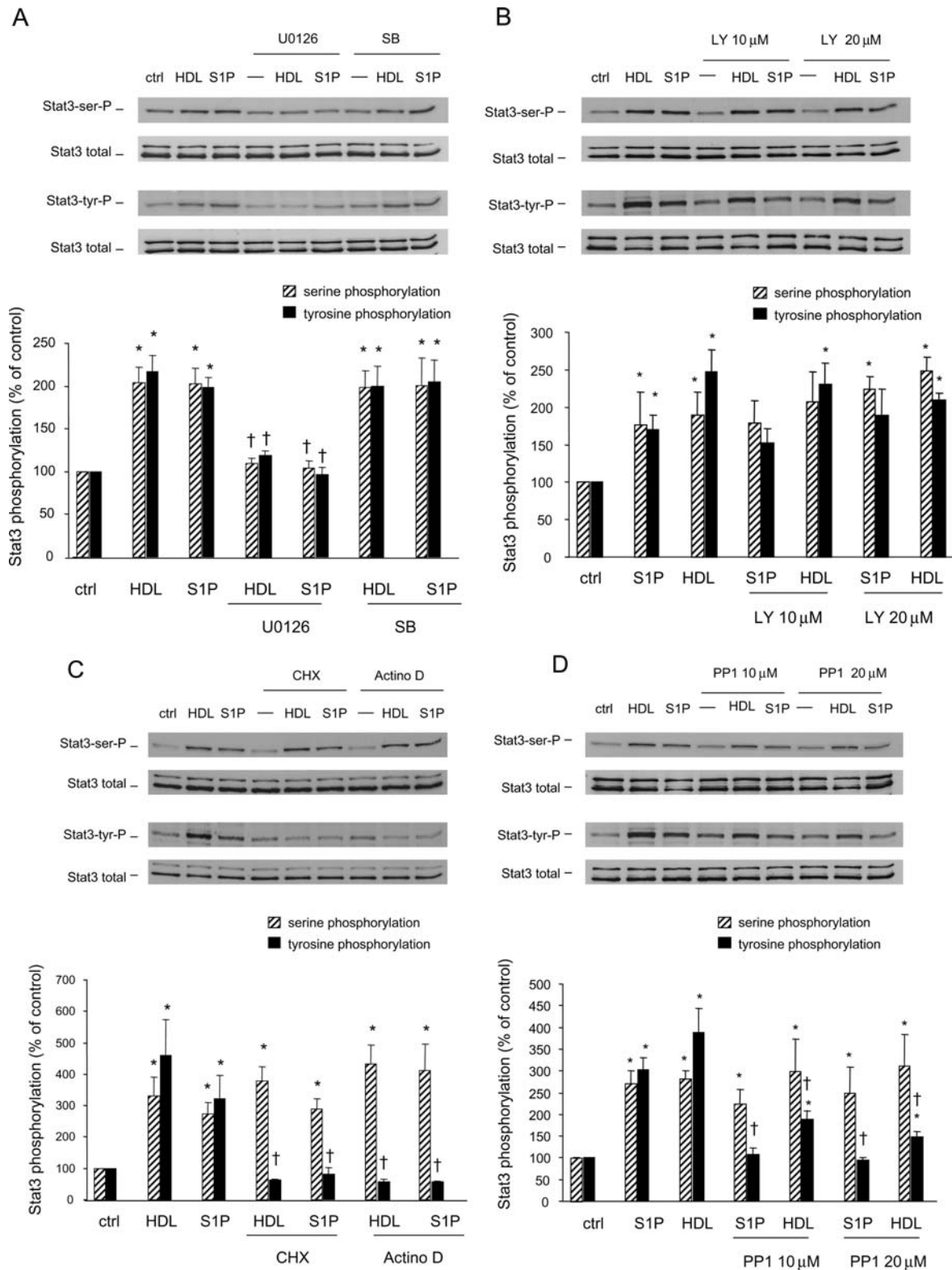
Our results indicate that ERK1/2 is involved in the Stat3 activation promoted by HDL or S1P, but neither p38 MAPK nor PI3K plays a role.

### 3.5 Rho-associated kinase and phospholipase C are involved in HDL- and sphingosine-1-phosphate-induced ERK1/2 and Stat3 phosphorylation

To clarify how HDL and S1P induce the activation of ERK1/2 and thus of Stat3, we pharmacologically inhibited ROCK, PLC, and PKC. In ventricular cardiomyocytes, the respective inhibitors of ROCK (Y27632,<sup>21</sup> 10  $\mu$ M) and PLC



**Figure 5** HDL and S1P induce ERK1/2 and p38 MAPK phosphorylation through S1P2. Cardiomyocytes were untreated (ctrl) or incubated with S1P antagonists, prior to stimulation for 90 min with S1P (1  $\mu$ M), S1P agonists (0.1–10  $\mu$ M), or HDL (400  $\mu$ g/mL). ERK1/2 and p38 MAPK phosphorylation (ERK1/2-P and p38 MAPK-P) was analysed by western blotting, as illustrated by representative western blots. Equal gel loading were assessed by total p38 MAPK. Specific bands corresponding to phosphorylated ERK1/2 and p38 MAPK were quantified by densitometry and expressed as percentage of the corresponding control. \* $P$  < 0.05 compared with control values,  $^{\dagger}P$  < 0.05 vs. S1P or HDL ( $n$  = 4). (A) Prior to stimulation with S1P or HDL, cells were treated for 20 min with 1 and 5  $\mu$ M of the S1P2 antagonist JTE013 (JTE). (B) Cardiomyocytes were stimulated with 0.1 and 1  $\mu$ M of S1P, SEW2871 (SEW, S1P1 receptor agonist), or FTY720 (FTY, S1P1, S1P3, S1P4, S1P5 receptor agonist). (C) Prior to stimulation with S1P or HDL, cells were treated for 20 min with 1 and 5  $\mu$ M of the S1P1/S1P3 antagonist, VPC23019 (VPC).



**Figure 6** HDL- and S1P-induced Stat3 activation is inhibited by U0126 and PP1 but not by SB203580 or LY294002. Cardiomyocytes were untreated (ctrl) or incubated with various signalling pathway inhibitors or with protein synthesis inhibitors, prior to stimulation for 90 min with S1P (1  $\mu$ M) or HDL (400  $\mu$ g/mL). Western blots: Specific bands corresponding to phosphorylated Stat3 were quantified by densitometry and expressed as percentage of the corresponding control. Equal gel loading were assessed by total Stat3. \* $P$  < 0.05 compared with control values,  $^{\dagger}P$  < 0.05 vs. S1P or HDL ( $n$  = 4). (A) Cells were left untreated (ctrl) or incubated for 30 min with 10  $\mu$ M of U0126 (U0) or SB203580 (SB), the inhibitors of ERK1/2 and p38 MAPK, respectively, prior to stimulation with HDL or S1P. (B) Cells were incubated for 1 h with 10  $\mu$ M and 20  $\mu$ M LY294002 (LY), the inhibitor of PI3K, prior to stimulation with S1P or HDL. (C) Cells were incubated for 20 min, with the translation inhibitor cycloheximide (CHX, 10  $\mu$ g/mL) or the transcription inhibitor actinomycin D (Actino D, 2.5  $\mu$ g/mL) prior to stimulation with S1P or HDL. (D) Cells were incubated for 1 h with 10  $\mu$ M and 20  $\mu$ M of PP1, the inhibitor of Src, prior to stimulation with S1P or HDL.



(U73122, 2  $\mu$ M) both partially reduced HDL and S1P-induced ERK1/2 phosphorylation as well as that of Stat3 on serine and tyrosine (see Supplementary material online, *Figures S1 and S2*). In contrast, the PKC inhibitor Ro-318220<sup>21</sup> (1  $\mu$ M) had no effect on the HDL and S1P-induced phosphorylation of ERK1/2 and Stat3 serine but partially inhibited that of Stat3 tyrosine (see Supplementary material online, *Figure S3*). Our results suggest the partial involvement of ROCK and PLC, but not PKC, in the activation of ERK1/2 induced by HDL and S1P.

### 3.6 Src is involved in HDL- and sphingosine-1-phosphate-induced tyrosine phosphorylation of Stat3

Having observed that HDL- and S1P-induced Stat3 tyrosine phosphorylation and Stat3 DNA-binding is delayed, mainly occurring after 60 min of stimulation, we hypothesized that newly synthesized proteins are necessary for Stat3 activation. Therefore, we blocked translation and transcription with cycloheximide and actinomycin D, respectively. As illustrated in *Figure 6C*, both inhibitors abolished HDL and S1P-induced Stat3 phosphorylation on tyrosine without affecting that on serine. Similarly, inhibition of the tyrosine kinase family Src with PP1<sup>21</sup> abolished Stat3 phosphorylation on tyrosine promoted by S1P and caused  $\sim 70\%$  reduction of the HDL-induced Stat3 phosphorylation. In contrast, PP1, had no effect on serine phosphorylation of Stat3 (*Figure 6D*). These findings were confirmed by experiments using the Src tyrosine kinase inhibitor 1 (see Supplementary material online, *Figure S4A*). Consistently, HDL and S1P were both found to induce a significant, transient increase in Src phosphorylation after 1 h of stimulation (see Supplementary material online, *Figure S4B*).

Our results indicate that newly synthesized protein(s) are necessary for HDL- and S1P-induced Stat3 activation and that tyrosine kinase family Src is involved in the tyrosine phosphorylation of Stat3.

## 4. Discussion

Our data show, for the first time to our knowledge, that HDL induces the activation of Stat3, and attribute a predominant role to S1P. Stat3 activation, as characterized by increased tyrosine phosphorylation and DNA-binding activity after stimulation of ventricular cardiomyocytes with HDL, was delayed when compared with cytokine-induced activation of Stat3 which is known to occur very rapidly in various cell types. In contrast, Stat3 phosphorylation on serine residues, which has been reported to play a modulatory role in the activation of Stat3,<sup>12,13</sup> occurred very rapidly after stimulation with HDL or S1P.

S1P and rHDLs containing S1P had a strong stimulatory action on Stat3, ERK1/2, and p38 MAPK, comparable to HDL isolated from human plasma. In contrast, rHDL containing apoA1 but no S1P had a much weaker effect, indicating that HDL-induced activation of Stat3, ERK1/2, and p38 MAPK occurs mainly through its S1P component. Confirming this hypothesis, the stimulatory effects of rHDL containing S1P and apoA1 were not significantly different from those of rHDL containing only S1P. In this context, it is important to note that rHDL containing S1P and apoA1 could lead to an increased cardioprotection by two different mechanisms. On the one hand, S1P and S1P-containing rHDL were found to

activate potent intracellular signalling pathways being involved in cardiac remodelling.<sup>2,8</sup> On the other hand, apoA1 and mimetic peptides have been shown to influence reverse cholesterol transport and reduce atherosclerosis in mice.<sup>22</sup>

Extracellular S1P acts by binding to plasma membrane G protein-coupled receptors identified as S1P1, S1P2, S1P3, S1P4, and S1P5. To date the S1P receptor subtypes that have been found to play a functional role in the cardiovascular system are S1P1, S1P2, and S1P3.<sup>23</sup> Using various agonists and antagonists of the G-protein-coupled S1P receptor subtypes S1P1–S1P5, including two potent and specific inhibitors of S1P1/S1P3 and S1P2, respectively, we found that S1P and HDL activate Stat3 mainly through the S1P2 receptor. In the case of HDL, the involvement of another receptor, possibly activated through the apoA1 component of HDL cannot be excluded. The observation that HDL and S1P-induced Stat3 activation was not affected by PTX suggests the involvement of a PTX-insensitive G protein such as G<sub>12/13</sub>, which has been shown to be coupled to the S1P2 receptor.<sup>18</sup> At the present time, there are few studies concerning the role of S1P receptors in cardiomyocytes. Robert *et al.*<sup>24</sup> reported that S1P induces cardiomyocyte hypertrophy mainly via the S1P1 receptor and subsequently via G<sub>i</sub> through ERK1/2, p38 MAPK, PI3K, and via Rho pathways. Similarly, the protective effect of S1P during hypoxia in ventricular cardiomyocytes was found to require activation of the S1P1 receptor and the PTX-sensitive G<sub>i</sub> protein.<sup>19</sup> In contrast, Theilmeier *et al.*<sup>8</sup> found that HDL and its constituent S1P protects the heart against I/R injury *in vivo* via an S1P3 receptor-mediated and NO-dependent pathway. In the same context, Means *et al.*<sup>25</sup> reported that both S1P2 and S1P3 receptors contribute to protect cardiomyocytes from I/R damage *in vivo*, and that the protective effect of S1P released in response to I/R involves receptor-mediated Akt activation. Clearly, further studies are necessary to elucidate whether in cardiac myocytes, S1P1, S1P2, and S1P3 receptors regulate different signalling pathways associated with specific functions.

The observation that HDL induces Stat3 activation in ventricular cardiomyocytes could be of clinical interest since there is increasing evidence that Stat3 is beneficial for the heart. Indeed, Stat3 appears to play diverse important roles in myocardium adaptation to stress; by maintaining myocardial capillarization, controlling interstitial collagen metabolism, protecting from apoptosis, and preserving cardiac function.<sup>9,10</sup> Conditional knockout mice harboring a cardiomyocyte-restricted deletion of Stat3 showed increased susceptibility to cardiac injury caused by myocardial ischaemia, inflammation, or drug toxicity.<sup>9,26</sup> Stat3 has also been shown to provide cardioprotection from the anti-tumour drug doxorubicin.<sup>26,27</sup> Moreover, it appears that Stat3 activation confers cardioprotection at the time of reperfusion in response to TNF- $\alpha$  and ischaemic preconditioning.<sup>28,29</sup>

There are two studies reporting a negative effect of HDL on Stat3 activation. Parhami *et al.*<sup>30</sup> found that exposure of osteoblast-like vascular cells to HDL for 24 h had no effect on the basal Stat3 phosphorylation level but partially inhibited interleukin-6-induced Stat3 phosphorylation. Similarly, Gharavi *et al.*<sup>31</sup> observed that in human aortic endothelial cells, HDL significantly reduced Stat3 tyrosine phosphorylation induced by oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine within 5 min while it had no effect on

basal Stat3 phosphorylation. These observations are not necessarily inconsistent with our results since we found that shorter time periods of exposition to HDL (3–30 min) resulted in a slight decrease in Stat3 tyrosine phosphorylation and that 5 min of stimulation had no effect on Stat3 DNA-binding. Indeed, we observed that HDL-induced Stat3 tyrosine phosphorylation and Stat3 DNA-binding is delayed, mainly occurring after 60–90 min of stimulation.

Our study revealed a role for ERK1/2 in HDL- and S1P-induced Stat3 activation. Indeed, inhibition of ERK1/2 but not of p38 MAPK or of PI3K abolished HDL-induced Stat3 activation. Consistent with these results, we have previously shown that ERK1/2 activation is a necessary event for prostaglandin E<sub>2</sub>-induced Stat3 activation in ventricular cardiomyocytes.<sup>14</sup>

The mechanism leading to the activation of ERK1/2 appears to be complex. Our findings indicate that ROCK and PLC but not PKC, account for at least part of the HDL and S1P-induced activation of ERK1/2. However, further studies are necessary to elucidate all the steps and crosstalk of the signalling cascades playing a role in the ERK1/2 activation in HDL-stimulated cardiomyocytes.

Blocking translation with cycloheximide showed that ERK1/2 plays an indirect role in the delayed tyrosine phosphorylation of Stat3 induced by HDL and S1P. Indeed, our results indicate that HDL and S1P-induced Stat3 activation requires newly synthesized protein(s), which could be or induce a non-receptor tyrosine-kinase. In agreement with this hypothesis, we found that in ventricular cardiomyocytes exposed to HDL or S1P for 90 min, the Src inhibitors PP1 and Src inhibitor 1 strongly reduced Stat3 phosphorylation on tyrosine but not on serine. These findings suggest that newly synthesized Src could play a role in HDL and S1P-induced Stat3 activation. However, at the present time, we cannot exclude that newly synthesized protein(s) could be released and act in an autocrine manner to stimulate a receptor-associated tyrosine kinase as shown for angiotensin II inducing Stat3 tyrosine phosphorylation through the autocrine action of secreted IL-6 family cytokines.<sup>32</sup> Further investigations are needed to elucidate the different steps involved in the delayed activation of Stat3 by HDL.

In summary, the present study demonstrates that in ventricular cardiomyocytes, HDL induces Stat3 activation mainly via its S1P constituent and the receptor S1P2. Moreover, we provide evidence for the involvement of ERK1/2 and Src in HDL-induced Stat3 activation. Our studies further indicate that ROCK and PLC are involved in the activation of ERK1/2 induced by HDL. These findings are in agreement with those of Hu *et al.*,<sup>33</sup> reporting that in smooth muscle, S1P2 but not S1P1 receptors activate PLC- $\beta$  and ROCK. The fact that rHDL containing S1P has the same effects as native HDL on Stat3 and ERK1/2, both known to play a role in cardiac remodelling, underlines the potential significance of modulating rHDL composition. The latter opens new perspectives of therapeutic strategies with rHDL tailored to confront particular pathological situations and offering increased cardioprotection.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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